Attach #22
Exhibit D

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 5888–5893, June 1996
Plant Biology

Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells

(plants/protoplasts/mutagenesis/reporter gene/blue fluorescent protein)

CHRISTOPH REICHEL*, JAIDEEP MATHUR, PETER ECKES†, KERSTIN LANGENKEMPER, CSABA KONCZ, JEFF SCHELL, BERND REISS, AND CHRISTOPH MAAS‡

Max-Planck-Institut für Züchtungsforschung, Abteilung Genetische Grundlagen der Pflanzenzüchtung, Carl-von-Linné Weg 10, D-50829 Köln, Germany; and †Hoechst-Schering AgrEvo GmbH, Forschung Biochemie H872N, Postfach 800320, D-65926 Frankfurt, Germany

Contributed by Jeff Schell, January 4, 1996

ABSTRACT **The expression of the jellyfish green fluorescent protein (GFP) in plants was analyzed by transient expression in protoplasts from *Nicotiana tabacum*, *Arabidopsis thaliana*, *Hordeum vulgare*, and *Zea mays*. Expression of GFP was only observed with a mutated cDNA, from which a recently described cryptic splice site had been removed. However, detectable levels of green fluorescence were only emitted from a small number of protoplasts. Therefore, other mutations in the *GFP* cDNA leading to single-amino acid exchanges in the chromophore region, which had been previously studied in *Escherichia coli*, were tested in order to improve the sensitivity of this marker protein. Of the mutations tested so far, the exchange of GFP amino acid tyrosine 66 to histidine (Y66H) led to detection of blue fluorescence in plant protoplasts, while the exchange of amino acid serine 65 to cysteine (S65C) and threonine (S65T) increased the intensity of green fluorescence drastically, thereby significantly raising the detection level for GFP. For GFP S65C, the detectable number of green fluorescing tobacco (BY-2) protoplasts was raised up to 19-fold, while the fluorimetricly determined fluorescence was raised by at least 2 orders of magnitude.**

A powerful tool for the rapid analysis of promoters is the use of marker genes for which expression can be easily monitored by autoradiography (*NPT II*, *CAT*), light emission (*LUC*, *GUS*), or color production (*GUS*). Commonly used reporter genes are *CAT*, *NPT II*, *GUS*, and *LUC*, of which *GUS* and *LUC* are of special interest since their assays do not involve any radioactivity (1). However, none of these reporter genes allows convenient, noninvasive *in vivo* detection of the respective enzyme in intact plant cells. An attractive alternative turned out to be the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. Use of this marker protein has been described for *Escherichia coli*, *Caenorhabditis elegans*, *Drosophila melanogaster*, yeast, and HeLa cells (2–5). Detection of GFP is noninvasive and nondestructive, which is a clear advantage over formerly used reporter genes such as β-glucuronidase or firefly luciferase (6, 7). Illumination of GFP with long-wave UV light (395 nm) or blue light (475 nm) leads to bright green fluorescence (510 nm) without any need for additional substrates, since chromophore formation and light emission are intrinsic properties of this marker protein (8).

Expression and detection of wild-type GFP in maize and sweet orange (*Citrus sinensis*) protoplasts using constructs driven by a heat-shock promoter or by a constitutive promoter (9–11), using a potato virus X expression system in *Nicotiana clevelandii* and *Nicotiana benthamiana* plants (12, 13), or using tobacco mosaic virus in *N. benthamiana* and tobacco protoplasts (14, 15), has been described recently. However, in our hands expression of a wild-type *GFP* cDNA driven by a *CaMV* 35S promoter was neither detectable in transgenic tobacco plants nor in transient expression studies in protoplasts from *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Hordeum vulgare*. Recently it has been described that the *A. victoria* wild-type *GFP* mRNA can be aberrantly processed in plant cells, due to the recognition of internal cryptic splice sites leading to inefficient expression of the GFP. Mutation of a cryptic splice site improves expression of GFP in cells from transgenic *A. thaliana* plants significantly (J. Haseloff, K. Siemering, D. Prasher, and S. Hodge, personal communication; ref. 16). Moreover, mutagenesis of the *GFP* cDNA in *Escherichia coli* has led to changes in the fluorescence properties of this new marker protein. A number of GFP amino acid exchange mutants have been isolated. They exhibit modifications in their excitation and emission spectra (17–21). Of these reported mutations, at least three are of great interest for expression in plant cells. *GFP* mutation Y66H (19) was shown to have a shifted emission peak leading to blue fluorescence. Analysis of a second set of *GFP* mutations, S65C and S65T (20, 21), revealed increased excitation and emission values, which might significantly improve brightness of green fluorescence also in plant cells.

In order to evaluate marker gene expression in plants, plant protoplasts provide a powerful tool. Transient gene expressi n studies in protoplasts from a variety of plant species and organs have been used widely as a rapid and powerful method to monitor gene expression and to analyze expression levels of different marker gene constructs (22–24). The investigation of several GFP derivatives by transient gene expression in protoplasts from widely used plant species would thus provide valuable information about the potential of mutations in the *GFP* cDNA to improve the brightness of green fluorescence and to alter the GFP emission spectrum.

Here we report transient expression studies of *GFP* genes, driven by the *CaMV* 35S promoter, in protoplasts of various mono- and dicotyledonous plant species. The cDNA of these *GFP* constructs was mutated to abolish aberrant splicing in plant cells. Additionally, this cDNA was modified to introduce single amino acid changes into the GFP chromophore regi n, leading to significantly improved brightness of green fluores-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GFP, green fluorescent protein; CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyltransferase; GUS, β-glucuronidase; LUC, firefly luciferase; NPT II, neomycin phosphotransferase II.

*To whom reprint requests should be addressed. e-mail: reichel@mpiz-koeln.mpg.de.
‡Present address: Hoechst-Schering AgrEvo GmbH, Forschung Biochemie H872N, Postfach 800320, D-65926 Frankfurt, Germany.

cence (S65C and S65T) or to the emission of blue fluorescence (Y66H) when expressed in plant cells.

MATERIALS AND METHODS

Cloning Strategies. All plasmids used in this study are based on a commercially available *GFP* cDNA (pGFP-1; Clontech) carrying a *NcoI* restriction site at the translation start codon and a modified 3′ end leading to the addition of four amino acids. By site-directed mutagenesis, an internal *NcoI* restriction site was destroyed (oligo5435: CCTGTTCCTTGGCCAACAC), and the wild-type stop codon was restored along with the introduction of *BamHI*, *BglII*, *XbaI*, and *AvrII* restriction sites 3′ of the new stop codon (oligo5618: CTATACAAATAAGGATCCAGATCTAGAATCCTAGGC). Plasmid pCRGFP was constructed by inserting the modified *GFP* cDNA as a *NcoI/BamHI* fragment into the plant expression vector pRTL2 GUS/NIa[Δ]Bam (25), thereby removing the *Gus/NIa* cassette and placing the modified *GFP* cDNA between the *CaMV* 35S promoter with a duplicated transcriptional enhancer, a tobacco etch virus translational enhancer and the *CaMV* 35S polyadenylation signal (Fig. 1). Plasmid pCKGFP 10 was constructed by exchanging an internal *NdeI/AccI* restriction fragment with the corresponding fragment from plasmid pBIN 35S-mGFP4, thus supplying the mutations for the removal of an internal cryptic splice site found in the wild-type *GFP* cDNA (J. Haseloff, K. Siemering, D. Prasher and S. Hodge; personal communication) (Fig. 1). Plasmids pCKGFP S65C, pCKGFP S65T, and pCKGFP Y66H were created by site-directed mutagenesis of plasmid pCKGFP 10 introducing single amino acid exchanges S65C (oligo5637: CTACTTTCTGTTATGGTGTACAATGC), S65T (oligo5858: CTACTTTCACTTATGGTGTACAATGC), and Y66H (oligo5652: CTACTTTCTCTCATGGTGTACAATGC). Plasmid pCKGFP S65Cmono contains the modified *GFP* cDNA from pCKGFP S65C driven by the *CaMV* 35S promoter and harboring the *Shrunken-1* exon 1/intron 1 sequences shown to significantly enhance reporter gene activity in monocot cells (22). During the course of our experiments, DNA sequence analysis of pGFP-1 (Clontech) directly from the originally supplied plasmid DNA, revealed a previously unreported point mutation in the *GFP* cDNA of this plasmid, leading to amino acid exchange I161T. The wild-type sequence (26) was restored by site-directed mutagenesis of the plasmids pCKGFP S65T, pCKGFP S65C(mono), and pCKGFP Y66H (oligo5653: GAATGGAATCAAAGTCAACTTCAA).

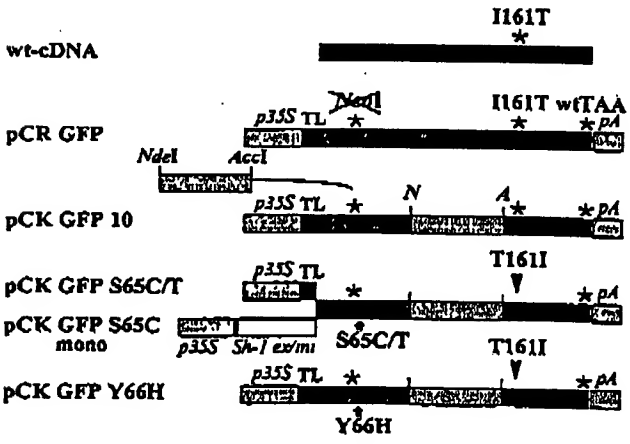

FIG. 1. Cloning strategies for GFP plant expression vectors. The wild-type *GFP* cDNA (pGFP-1) was modified and introduced into a plant expression vector to give plasmid pCRGFP. Insertion of a *NdeI–AccI* restriction fragment, containing murations in order to delete a cryptic splice site, led to plasmid pCKGFP 10. By site-directed mutagenesis of pCKGFP 10, chromophore amino acids 65, (Ser → Cys) and (Ser → Thr), and 66 (Tyr → His) were exchanged, leading to plasmids pCKGFP S65C, pCKGFP S65T and pCKGFP Y66H. pCKGFP S65Cmono was constructed by insertion of *GFP* coding region from pCKGFP S65C into a monocot expression vector. TL, translational enhancer; wt, wild-type; *, point mutations.

Protoplast Transfection Protocols. Preparation and PEG-mediated DNA uptake with *A. thaliana* mesophyll protoplasts was performed as described in Mathur *et al.* (27). Transfection of tobacco and barley protoplasts followed protocols from Negrutiu *et al.* (28) and Maas *et al.* (23), respectively. Electroporation of mesophyll protoplasts from etiolated maize seedlings isolated essentially according to Sheen (29) was performed with a Dialog Electroporator II (Düsseldorf, Germany). Electroporation conditions were 500 V/cm and 200 ms. Each sample contained 3×10^5 protoplasts and 40 μg of plasmid DNA in 0.3 ml of 0.8 M mannitol and 20 mM KCl. After electroporation, the protoplasts were cultivated in 1 ml of 0.8 M mannitol and 10 mM Mes (pH 5.7) for 20 h at room temperature in the dark prior to microscopical analysis. Protoplasts from other species were analyzed by fluorescence microscopy 48 h after transfection.

Microscopic Analysis. Microscopic studies were performed using an Aristoplan fluorescence microscope (Leitz, Germany). For fluorescence studies, filter blocks A (UV light exciter BP 340–380 nm; beamsplitter RKP 400 nm; emitter LP 430 nm) and I3 (blue light exciter BP 450–490 nm; beamsplitter RKP 510 nm; emitter LP 520 nm) were used (Leitz, Germany). For the elimination of chlorophyll autofluorescence in tobacco SR1 mesophyll protoplasts, the filter set 41014 (exciter HQ 450/50; beamsplitter Q 480 LP; emitter HQ 510/50) was used (Chroma Technology). Experiments were documented using Kodak Ektachrome 320T and Kodak Ektachrome Panther P1600x films.

Fluorimetry. For the fluorimetric measurement of GFP green fluorescence, tobacco BY-2 protoplasts were transfected with plasmids pCKGFP 10 and pCKGFP S65C. Viable and dead protoplasts were separated 24 h after transfection by centrifugation (3,300 × *g* for 20 min) through 33% Percoll. Viable protoplasts were collected at gradient top and disrupted by six passages through an 18-gauge needle. Extracts were diluted 10-fold in 50 mM sodium phosphate buffer (pH 7), and an equivalent of approximately 50,000 protoplasts was measured in a filter fluorimeter (LS-2B, Perkin-Elmer) using excitation filter 139168 (480 nm) for blue light excitation. The emission spectra were scanned between 495 and 540 nm, and the emission spectrum determined for a mock-transfected tobacco BY-2 sample was substracted from the spectra determined for transfections with plasmids pCKGFP 10 and pCKGFP S65C.

RESULTS AND DISCUSSION

Several plasmids containing the *GFP* cDNA driven by the *CaMV* 35S promoter were tested in transient gene expression studies in protoplasts derived from suspension culture cells as well as leaf mesophyll. Protoplasts were transfected using PEG-mediated gene transfer or electroporation (maize), and GFP expression was monitored 20–48 h after transfection by the detection of green fluorescence using fluorescence microscopy.

Transient expression of GFP in protoplasts from tobacco BY-2 suspension culture resulted in bright green fluorescence (Fig. 2 *a* and *b*). However, green fluorescence was only detected with a modified *GFP* cDNA, from which a cryptic splice site had been removed (Fig. 2*a*; plasmid pCKGFP 10). This modification leads to significantly increased expression of GFP in transgenic *A. thaliana* (J. Haseloff, K. Siemering, D. Prasher, and S. Hodge; personal communication). With a *GFP* cDNA (Clontech), which had not been engineered for the

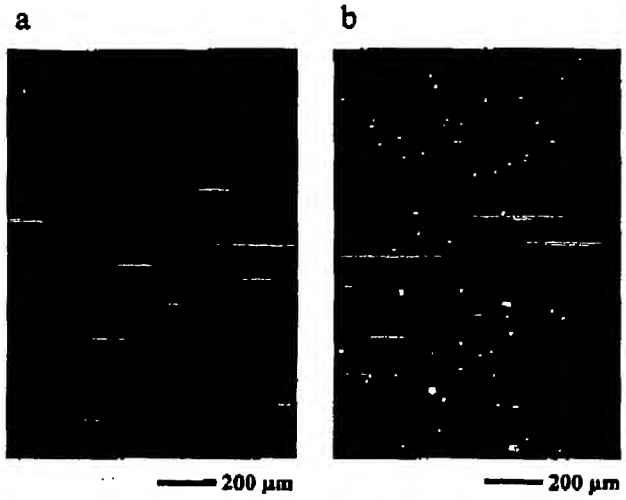

FIG. 2. Analysis of GFP expression in *N. tabacum* suspension culture protoplasts (BY-2). Comparison of constructs pCKGFP10 and pCKGFP S65C. Protoplasts were viewed at low magnification under blue light excitation (450–490 nm). (*a*) pCKGFP 10; (*b*) pCKGFP S65C. Transfection efficiencies, as judged by fluorimetric measurement of GUS activity derived from co-transfection with plasmid pRTL2 GUS (25), were nearly identical in the two experiments (data not shown).

removal of the cryptic splice site, we failed to detect any green fluorescence in *A. thaliana* and tobacco SR1 mesophyll protoplasts, in protoplasts from barley and tobacco BY-2 suspension culture cells (plasmid pCRGFP), as well as in transgenic tobacco plants (data not shown). This is in agreement with Haseloff and Amos (16), demonstrating that these modifications are also essential for an efficient detection of green fluorescence in transgenic *A. thaliana* plants.

Although green fluorescence of GFP using the cDNA, from which a cryptic splice site had been removed (pCKGFP 10), was observed in all protoplast species after blue light excitation [parsley, *Arabidopsis*, maize, barley, and tobacco SR1 (data not shown) and tobacco BY-2 protoplasts (Fig. 2*a*)], the brightness of green fluorescence was low and detection was therefore only possible in a small number of protoplasts. Therefore, we set out to further improve the brightness of GFP fluorescence in plant cells. Previously, several *GFP* mutants had been analyzed in *E. coli*, some of which showed drastically shifted excitation and emission spectra (17–21). Of particular interest for us were *GFP* mutants S65C and S65T. Heim *et al.* (20) demonstrated for GFP S65C and S65T about 6-fold higher excitation and emission values after illumination with blue light. These characteristics should lead to increased brightness of fluorescence in plants, making *GFP mutants S65C and S65T attractive candidates for plant studies. Therefore the GFP* cDNA, from which a cryptic splice site had been removed, was modified by site-directed mutagenesis so as to replace amino acid serine 65 with cysteine (S65C) and threonine (S65T). Viewed at low magnification (50×), transient expression studies with *GFP* mutant S65C (and S65T, data not shown) in tobacco BY-2 protoplasts showed a dramatic increase in the number and brightness of green fluorescing protoplasts (Fig. 2*b*). The number of detectable, green fluorescing protoplasts was increased up to 19-fold for GFP S65C (up to 7-fold for GFP S65T) when compared with transfections with pCKGFP 10 (Table 1, Fig. 2). Co-transfection with a chimeric *CaMV* 35S-GUS reporter gen (data not shown) revealed nearly identical transfection rates for these experiments. Therefore, when comparing Fig. 2 *a* and *b*, which represent identical microscopic and phot graphic conditions, the increase in the number, as well as the brightness of individual fluorescing

Table 1. Comparison of the detectable number of green fluorescing tobacco BY-2 protoplasts after transfection with different GFP constructs

GFP construct	Number of counted protoplasts	% green flu rescing protoplasts	Increase (fold after GUS normalization)
pCKGFP10	27985	1.1	1
pCKGFP S65C	16785	9.9	10–19
pCKGFP S65T	13196	8.2	6–7

Viable and green fluorescing protoplasts from three independent experiments were counted. After normalization with an internal standard (co-transfection with the reporter gene GUS), the factor by which the detectable number of green fluorescing protoplasts was increased, has been calculated.

protoplasts, suggests that GFP S65C is detectable in many protoplasts in which GFP alone is not. Fluorimetric measurement of green fluorescence from tobacco BY-2 protoplasts transfected with plasmids pCKGFP 10 or pCKGFP S65C showed that the intensity of green fluorescence with GFP S65C was raised by at least 2 orders of magnitude (Fig. 3). Dramatically increased brightness of individual protoplasts and an overall increase in the number of strongly fluorescing protoplasts was also observed with mesophyll and suspension culture protoplasts from *N. tabacum* and *A. thaliana* in transient expression experiments with plasmid pCKGFP S65C (data not shown).

Our observations here, that it is necessary to use the *GFP* cDNA from which the cryptic splice site had been removed (pCKGFP 10), contrast with previous observations of GFP expression in maize and *Citrus* plant cells using the wild-type *GFP* cDNA (9–11, 30). We found no expression of pCR GFP (Fig. 1; Clontech "wild-type"-cDNA) in *Arabidopsis*, tobacc , and barley protoplasts (data not shown). This may reflect differences in the splice mechanisms of maize and *C. sinensis* suspension cultures versus *N. tabacum*, *A. thaliana*, and *H. vulgare*. In any case, we find that a cDNA encoding GFP S65C, from which the cryptic splice site has been removed, results in clearly enhanced green fluorescence after blue light excitation also in maize protoplasts, as in other plant protoplasts. Approximately 30% of the viable maize protoplasts emitted green light (Fig. 4*d*; data not shown).

Bright green fluorescence was also previously observed using the wild-type *GFP* cDNA in *N. clevelandii*, *N. benthami-*

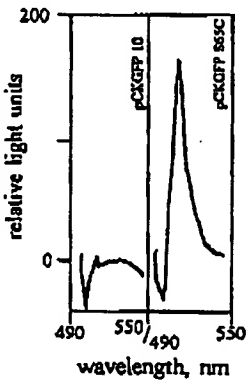

FIG. 3. Fluorimetric measurement of GFP green fluorescence in tobacco BY-2 protoplasts. Extracts of mock-transfected tobacco BY-2 protoplasts and of protoplasts transfected with plasmids pCKGFP 10 and pCKGFP S65C were measured in a filter fluorimeter. The spectrum determined for the mock-transfected protoplasts was substracted from the spectra measured for pCKGFP 10 and pCKGFP S65C to eliminate background fluorescence. Left chart sh ws spectrum for pCKGFP 10. Only a small peak at 510 nm is visible. Right chart shows spectrum for pGKGFP S65C, with a strong peak at 510 nm.

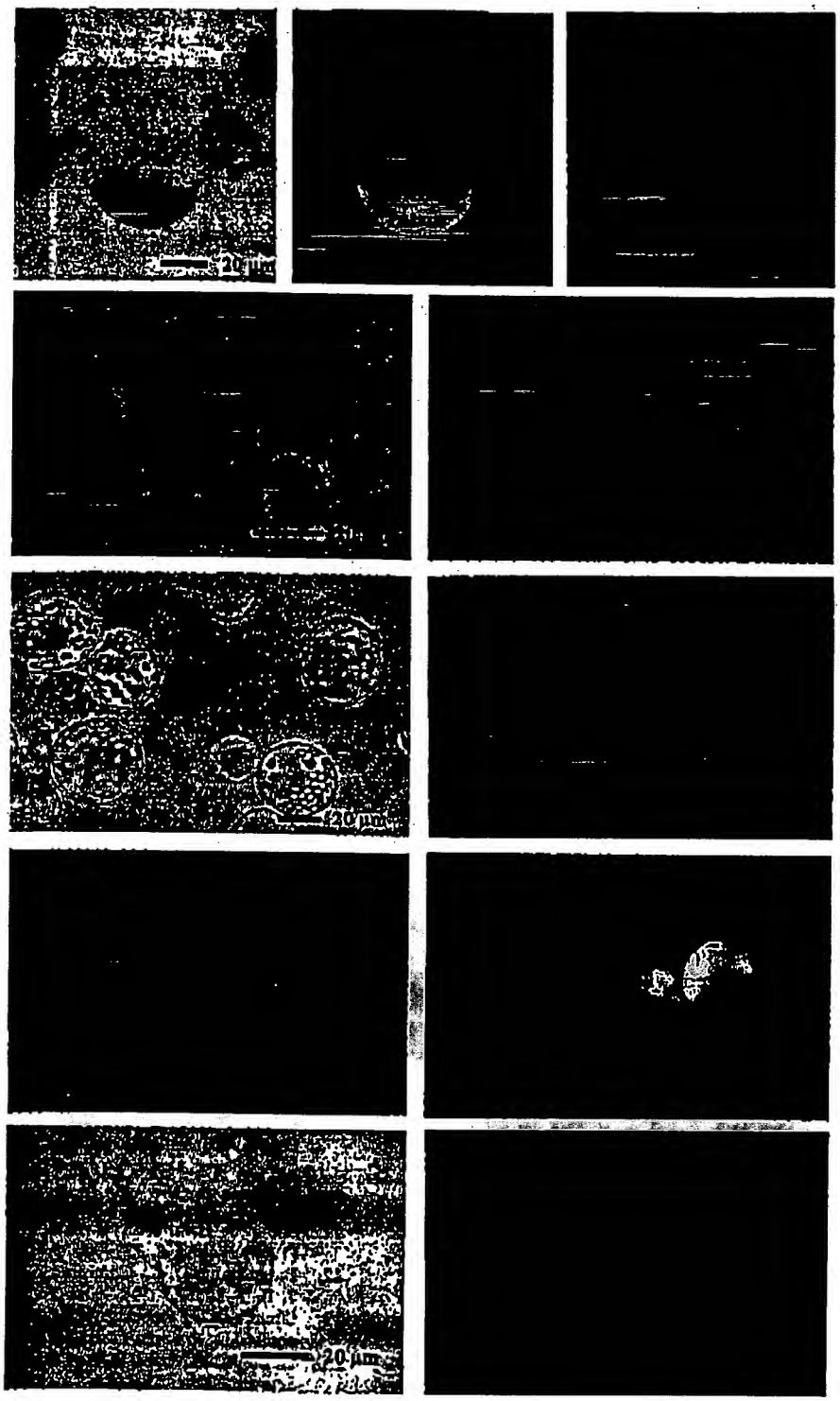

FIG. 4. Transient expression of plasmids pCKGFP S65C (*a–d*) or pCKGFP S65Cmono (*e*) in protoplasts from tobacco SR1 (mesophyll) (*a*), tobacco BY-2 (suspension) (*b*), *A. thaliana* (mesophyll) (*c*), *Z. mays* (mesophyll) (*d*), and *H. vulgare* (suspension) (*e*). Protoplasts were viewed with blue light excitation (450–490 nm; right row) and in bright field (left row). Tobacco SR1 mesophyll protoplasts were viewed with filter set I3 (*a* middle part, blue light excitation) and with GFP filter set 41014 which blocks the red autofluorescence from chlorophyll (*a* right part).

ana, and tobacco protoplasts, using virus-based expression systems (12–15). For infections with RNA transcripts, the eukaryotic splice machinery is most likely circumvented, since potato virus X and tobacco mosaic virus are believed to replicate in the cytoplasm of the infected plant cells (31). After DNA inoculation, transcription of plasmid DNA and inefficient splicing of the potato virus X RNA in *N. clevelandii* and *N. benthamiana* may have led t survival of unspliced viral RNA, which after transport to the cytoplasm served as replication template, thus leading to detectable levels of bright green fluorescence.

In subsequent experiments we used the *GFP* cDNA exclusively, from which the cryptic splice site had been removed, combined with mutation S65C, either under control of the *CaMV* 35S promoter for expression in dicotyledonous cells, or optimized for expression in monocot cells by combination with the *Shrunken-1* intron 1 and exon 1 (22). The excitation maximum (479 nm) of this particular mutant lies within the limits of the used fluorescein isothiocyanate filter set (exciter BP 450–490 nm), allowing an optimal excitation of GFP S65C, while the range of this filter set is already suboptimal for the excitation maximum of GFP S65T (488 nm). This also may explain why *GFP* mutant S65T in our hands leads to a slightly lower increase (6–7-fold) in the number of detectable green fluorescing protoplasts, when compared with GFP S65C (up to 19-fold; Table 1).

In protoplasts from tobacco SR1 and BY-2 (Fig. 4 *a* and *b*), *A. thaliana* (Fig. 4*c*), *Zea mays* (Fig. 4*d*), and *H. vulgare* (Fig. 4*e*), bright green fluorescence was observed in transient expression experiments with the respective plasmids designed for monocotyledonous (pCKGFP S65Cmono; Fig. 1) or dicotyledonous plant cells (pCKGFP S65C; Fig. 1). Green fluorescence of GFP in tobacco SR1 mesophyll protoplasts was initially somewhat obscured by the strong red autofluorescence of chlorophyll after illumination with blue light. Nevertheless, in tobacco mesophyll protoplasts expressing high levels of GFP or with chloroplasts out of focus or concentrated in one half of the cell, detection of bright green fluorescence was clearly possible (Fig. 4*a* middle panel). To reduce problems due to autofluorescence of chloroplasts, we subsequently used filtersets, specially designed for GFP detection, that block red light without affecting green fluorescence derived from GFP expression. In the tobacco mesophyll protoplast, the strong background of red chlorophyll fluorescence (Fig. 4*a* middle panel) was clearly reduced when using filter set 41014 (Fig. 4*a* right panel).

Alteration of GFP amino acid 66 (Y>H) has been shown to lead to blue fluorescence in *E. coli* when illuminated with UV light (19). This is the only reported example of a *GFP* mutation with a significant shift of the emission wavelength from green (510 nm) to blue (448 nm). Additionally the secondary excitation peak at wavelength 475 (blue light) is lost, leaving UV light excitation nearly unchanged (wild-type, 395 nm, to Y66H, 382 nm). The *GFP* cDNA, from which the cryptic splice site had been removed, was engineered by site-directed mutagenesis to alter amino acid tyrosine 66 to histidine (Y66H). Transient expression analysis in tobacco BY-2 suspension protoplasts illuminated with long-wave UV light revealed blue fluorescing cells (Fig. 5). These blue fluorescing cells were easily distinguishable from dead and damaged cells, which showed a very pale, cyan/"bluish" autofluorescence (data not shown). Although the blue fluorescence was clearly detectable (Fig. 5), signals vanished within short time, due to photobleaching by UV light. This is in contrast to GFP S65C (and GFP S65T), which even after several minutes of illumination with blue light showed no photobleaching.

In conclusion, improvement of GFP fluorescence by the S65C (and S65T) mutation, which leads to increased excitation and emission values and high photostability with blue light excitation, in combination with the elimination of the cryptic splice site, greatly increases the potential of the *A. victoria* green fluorescent protein as an alternative marker for nondestructive analysis of gene expression in plants. Additionally, blue fluorescence from *GFP* mutant Y66H may allow parallel analysis of different chimeric gene fusions by co-transformation and resolution of blue and green fluorescence coming from a single cell. Whether brightness and stability of blue fluorescence of GFP Y66H can be further enhanced by additional mutations remains to be determined.

We thank M. Kalda for photographic work and M. Nilges for excellent technical assistance. The authors are grateful to Dr. J. Haseloff for the generous gift of plasmid pBIN 35S-mGFP4 harboring the *GFP* cDNA, from which a cryptic splice site had been removed, and sharing experimental data prior to publication. This work was financed in part by the German-Israeli Foundation (I-0213-228.12/91).

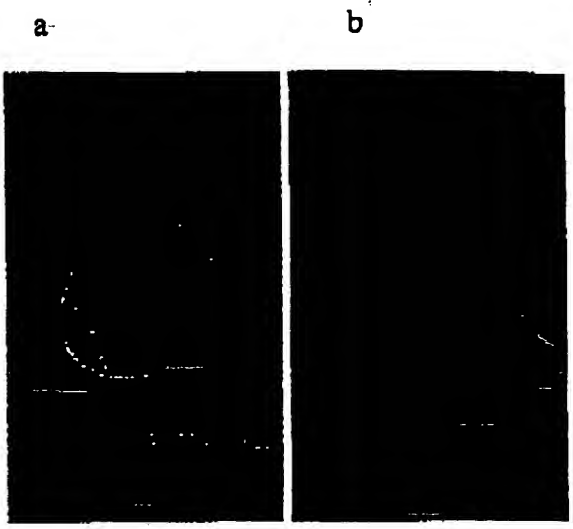

FIG. 5. Expression of blue fluorescent protein (Y66H) in a protoplast from BY-2 suspension culture. BY-2 protoplasts were transfected with plasmid pCKGFP Y66H and viewed with brightfield (*a*) and UV light excitation (*b*) (340–380 nm).

1. Suter-Crazzolara, C., Klemm, M. & Reiss, B. (1995) in *Methods in Cell Biology: Methods in Plant Cell Biology*, Part B, eds. Galbraith, D. W., Bourque, D. P. & Bohnert, H. J. (San Diego: Academic Press), Vol. 50, pp. 425–438.
2. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. (1994) *Science* 263, 802–805.
3. Wang, S. X. & Hazelrigg, T. (1994) *Nature (London)* 369, 400–403.
4. Corbett, A. H., Koepp, D. M., Schlenstedt, G., Lee, M. S., Hopper, A. K. & Silver, P. A. (1995) *J. Cell Biol.* 130, 1017–1026.
5. Kaether, C. & Gerdes, H.-H. (1995) *FEBS Lett.* 369, 267–271.
6. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. (1987) *EMBO J.* 6, 3901–3907.
7. De Wet, J. R., Wood, K. V., De Luca, M., Helinski, D. R. & Subramani, S. (1987) *Mol. Cell. Biol.* 7, 725–737.
8. Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. G. & Ward, W. W. (1993) *Biochemistry* 32, 1212–1218.
9. Galbraith, D. W., Lambert, G. M., Grebenok, R. J. & Sheen, J. (1995) in *Methods in Cell Biology: Methods in Plant Cell Biology*, Part B, eds. Galbraith, D. W., Bourque, D. P. & Bohnert, H. J. (San Diego: Academic Press), Vol. 50, pp. 3–14.
10. Niedz, R. P., Sussman, M. R. & Satterlee, J. S. (1995) *Plant Cell Rep.* 14, 403–406.
11. Sheen, J., Hwang, S., Niwa, Y., Kobayashi, H. & Galbraith, D. W. (1995) *Plant J.* 8, 777–784.
12. Baulcombe, D. C., Chapman, S. & Santa Cruz, S. (1995) *Plant J.* 7, 1045–1053.
13. Oparka, K. J., Roberts, A. G., Prior, D. A. M., Chapman, S., Baulcombe, D. & Santa Cruz, S. (1995) *Protoplasma* 189, 133–141.
14. Heinlein, M., Epel, B. L., Padgett, H. S. & Beachy, R. N. (1995) *Science* 270, 1983–1985.
15. McLean, B. G., Zupan, J. & Zambryski, P. C. (1995) *Plant Cell* 7, 2101–2114.
16. Haseloff, J. & Amos, B. (1995) *Trends Genet.* 11, 328–329.
17. Delagrave, S., Hawtin, R. E., Silva, C. M., Yang, M. M. & Youvan, D. C. (1995) *Bio/Technology* 13, 151–154.
18. Ehrig, T., O'Kane, D. J. & Prendergast, F. G. (1995) *FEBS Lett.* 367, 163–166.
19. Heim, R., Prasher, D. C. & Tsien, R. Y. (1994) *Proc. Natl. Acad. Sci. USA* 91, 12501–12504.
20. Heim, R., Cubitt, A. B. & Tsien, R. Y. (1995) *Nature (London)* 373, 663–664.

21. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A. & Tsien, R. Y. (1995) *Trends Biochem. Sci.* 20, 448–455.
22. Maas, C., Laufs, J., Grant, S., Korfhage, C. & Werr, W. (1991) *Plant Mol. Biol.* 16, 199–207.
23. Maas, C., Reichel, C., Schell, J. & Steinbiß, H.-H. (1995) in *Methods in Cell Biology: Methods in Plant Cell Biology*, Part B, eds. Galbraith, D. W., Bourque, D. P. & Bohnert, H. J. (San Diego: Academic Press), Vol. 50, pp. 383–399.
24. Fischer, R. & Hain, R. (1995) in *Methods in Cell Biology: Methods in Plant Cell Biology*, Part B, eds. Galbraith, D. W., Bourque, D. P. & Bohnert, H. J. (San Diego: Academic Press), Vol. 50, pp. 401–410.
25. Carrington, J. C., Freed, D. D. & Leinicke, A. J. (1991) *Plant Cell* 3, 953–962.
26. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G. & Cormier, M. J. (1992) *Gene (Amst.)* 111, 229–233.
27. Mathur, J., Koncz, C. & Szabados, L. (1995) *Plant Cell Rep.* 14, 221–226.
28. Negrutiu, I., Shillito, R., Potrykus, I., Biasini, G. & Sala, F. (1987) *Plant Mol. Biol.* 8, 363–373.
29. Sheen, J. (1990) *Plant Cell* 2, 1027–1038.
30. Hu, W. & Cheng, C.-L. (1995) *FEBS Lett.* 369, 331–334.
31. Matthews, R. E. F. (1991) *Plant Virology*. 3rd Ed., (San Diego: Academic Press).